A mechanical explanation of RNA pseudoknot function in programmed ribosomal frameshifting.

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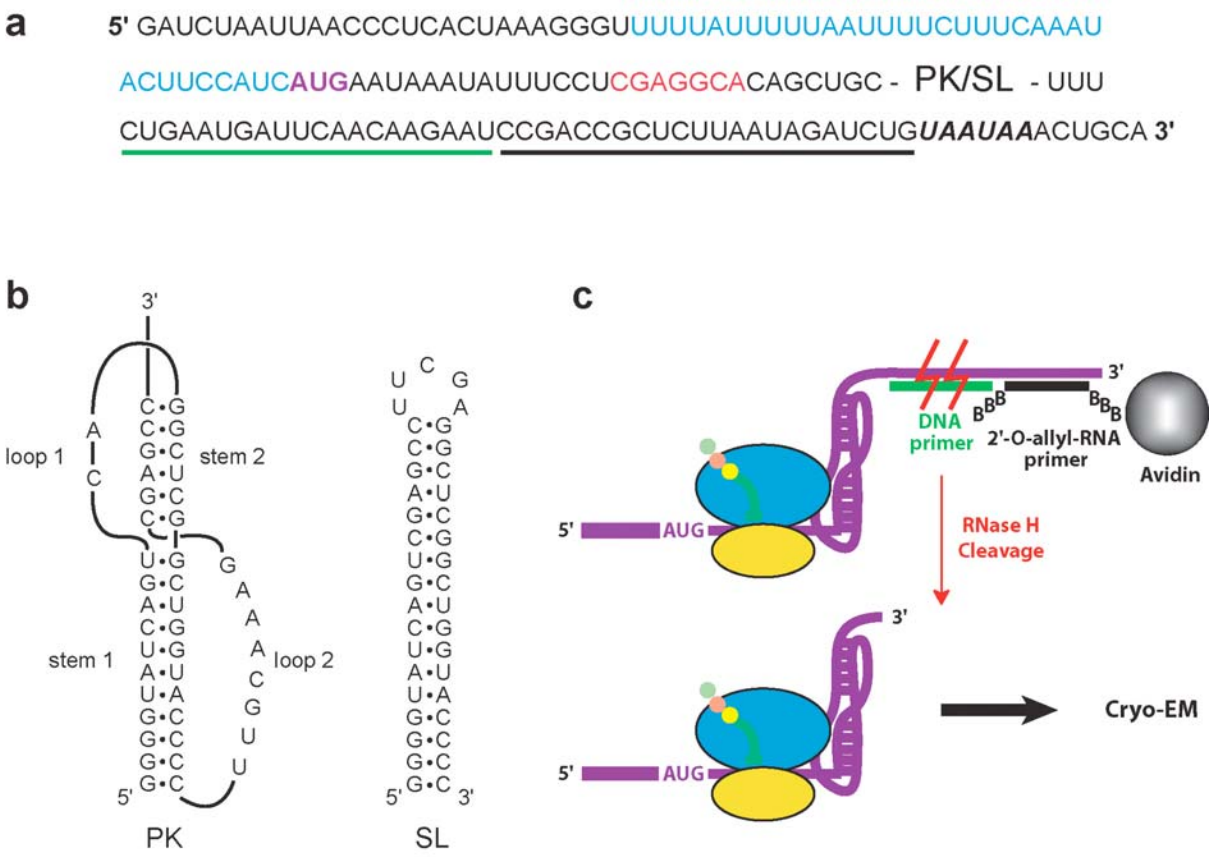
Supplementary Information

1. Supplementary Figure 1



Legend to Supplementary Figure 1

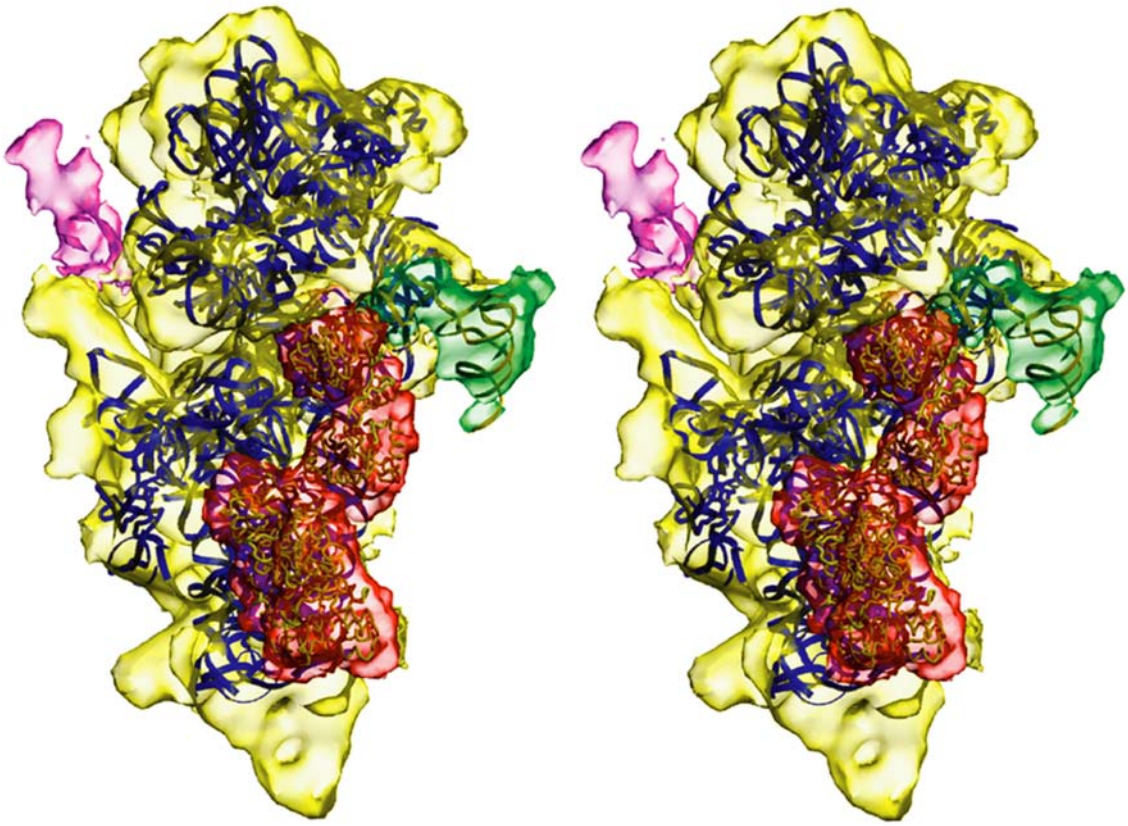
Preparation of stalled 80S complexes.

(a). Messenger RNAs for *in vitro* translation contained the alfalfa mosaic virus RNA 4 unstructured leader[30] (bases in blue), a start codon (AUG, purple), a pseudoknot (PK) or stem-loop (SL) and a downstream region containing sequences complementary to DNA (underlined in green) or 2'-O-allyl RNA (underlined in black) oligonucleotides. Tandem translation termination codons were present near the end of the mRNA (UAA, bold and italicised). Bases in red occupy the position of the natural infectious bronchitis virus (IBV) slippery sequence (UUUAAAC).

(b). The sequences of the minimal IBV pseudoknot (PK) and related stem-loop (SL) present in the mRNAs are shown.

(c) Following *in vitro* translation and pelleting through sucrose, stalled ribosomes were purified on an avidin matrix using a pre-annealed biotinylated 2'-O-allyl RNA oligonucleotide (black). Complexes were released by RNase H-directed cleavage using a complementary DNA primer (green) and subjected to cryo-EM. Molecules are not to scale.

Supplementary Figure 2



Legend to Supplementary Figure 2

A stereoview of the small subunit of the pseudoknot-stalled complex is shown viewed in the same orientation as Figure 1. The subunit, tRNA, eEF2 and pseudoknot are coloured as in Figure 1. The yeast atomic model for the small subunit[15] has been fitted to the subunit itself (blue ribbons), the structure of eEF2 (Ref. 18) to the corresponding density (yellow coil), and the tRNA stalled within the complex likewise (yellow ribbon).

2. Supplementary Methods

Isolation of paused 80S-mRNA complexes. Messenger RNAs (~190 nt) were derived from plasmid pEMC-PK or -SL and contained the minimal IBV pseudoknot[23] or related stem-loop structure[23] respectively. *In vitro* translations were carried out using nuclease-treated rabbit reticulocyte lysates (RRL; Promega). The relevant mRNA transcript (300pmole) was annealed to a chimeric biotinylated DNA/2' O-allyl modified RNA oligonucleotide (5' TbTbTbCAGAUCUAUUAAGAGCGGUCGGTbTbTb 3'; 800pmol) in a 10μl reaction containing 20mM Tris pH 7.5, 4mM $MgCl_2$ and 100mM KCl for 5 min at 68°C, 4 min at 37°C and 10 min on ice. The mRNA/oligonucleotide complex was added to 2.4ml of RRL and translation allowed to proceed for 15 min at 27°C prior to addition of cycloheximide (to 1mM). The translation reaction was diluted to 4 ml with HMCK buffer (25mM Hepes-KOH pH 7.8, 5mM MgOAc, 1mM cycloheximide, 150mM KOAc), loaded onto a cushion of sucrose (10ml 34% sucrose in HMCK), centrifuged at 440,000g for 1 hour at 4°C and the pellet resuspended gently in 900μl HMCK. A 1ml (settled bed volume) aliquot of avidin resin (SoftLink, Promega) was preadsorbed with biotin (10mM) and tRNA (100μg). Subsequently, the resin was preadsorbed with unprogrammed RRL (1ml) and BSA (1mg) before addition of the sucrose-purified ribosomes. After 30 min, the resin was washed (four times with 1 ml HMCK), removed from the column, collected by centrifugation (13,000g, 1 min) and resuspended in 250μl HMCK containing 1nmole of a DNA oligonucleotide (5'ATTCTTGTTGAATCATTCAG 3') complementary to mRNA sequences 3' of the pseudoknot/stem-loop. After incubation, mRNA/ribosome complexes were released from the SoftLink resin by addition of 30U RNaseH (Promega). The resin was pelleted (13,000g, 1 min), the supernatant retained and respun to remove all traces of resin. Ribosomes, final concentration 5 OD260/ml, were aliquoted and frozen at –70°C prior to cryo-EM.

Cryo-electron microscopy and image processing. Samples were blotted onto holey carbon-coated copper mesh microscope grids and vitrified by plunging into liquid ethane. Images were captured using a 200 kV Phillips CM200 FEG electron microscope under low dose

conditions and at a range of defocus values. The micrographs were scanned using a UMAX PowerLook 3000 scanner on an 8.322 μm raster and then interpolated to 16.64 micron, providing a pixel size at the specimen of 3.33A. Particles were excised and CTF-corrected using EMAN[31]. $80S_{Apo}$ particles were subjected to *ab initio* structure determination using angular reconstitution in IMAGIC[24]. Once initial maps were obtained, they were refined iteratively in SPIDER[25] by projection matching. The $80S_{Apo}$ was used as the alignment template for the stalled ribosome images, again by projection matching in SPIDER. Once initial reconstructions of the stalled ribosomes were generated, several rounds of iterative refinement followed. Alignments in SPIDER were initially on a 15º grid, iteratively refined to 5º. Reconstructions were improved during this process by using a cross-correlation function between each particle image and the corresponding reconstruction to select the best 50% of each set, and by three-way alignment with ranking by correlation coefficient. Next, local alignment procedures[32] were employed down to an angular spacing of 1º and maps were produced by weighting the contribution of individual particle images by the correlation coefficient calculated for them to the alignment model within a mask defined by the common presence of a P-site tRNA in both stalled reconstructions. Finally, pairwise alignment of each image of the $80S_{PK}$ and $80S_{SL}$ samples was carried out, each being aligned to the current-best model for that sample and to the $80S_{Apo}$ reconstruction. Images of $80S_{PK}$ or $80S_{SL}$ complexes were judged to be fully occupied if their correlation coefficient within a mask defined either by the P-site tRNA, eEF2 and pseudoknot (for $80S_{PK}$) or by the P-site tRNA (for $80S_{SL}$) was greater when correlated to the current-best complex map than to the $80S_{Apo}$ map; 12,161 out of 17,672 images of $80S_{PK}$ and 9,805 out of 14,887 images of $80S_{SL}$ were in this manner selected. Use of these selected images subsequently produced reconstructions in which tRNAs, eEF2 and pseudoknot displayed full and equal occupancy. The $80S_{Apo}$ map incorporated 10,296 images. The final data were divided into halves and compared using Fourier shell correlation (FSC) to give resolutions of 14.0 Å ($80S_{Apo}$), 15.7 Å ($80S_{SL}$) and 16.2 Å ($80S_{PK}$) at the $FSC_{0.5}$ cutoff. Maps were scaled in reciprocal space to evenly-spaced scattering centres computed within the unscaled maps, as previously described (programmes

GAP[32], SHELLSCALE and ISOSCALE (D.I.S., unpublished)), and sharpened by application of a B-factor of -500 Å^2.

Fitting of atomic structures. The atomic models composed by Spahn and colleagues for the yeast 40S and 60S subunits[15] were fitted to the 80S reconstructions in O[26] and refined using the program URO[27]. Models for eEF2 and tRNA were obtained from the Protein Data Bank, accession codes 1N0U (sordarin complex) and 2TRA (tRNA^{Asp}). EF2 was split manually into two domains and refined using URO. Figures were generated using BOBSCRIPT[28] and Raster3D[29].

3. Supplementary Data

Supplementary References

30. Jobling, S.A. & Gehrke, L. Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence. *Nature* **325**, 622-625 (1987).

31. Ludtke, S. J., Baldwin, P. R. & Chiu, W. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* **128**, 82-97 (1999).

32. Gilbert, R. J. *et al.* Three-dimensional structures of translating ribosomes by Cryo-EM. *Mol. Cell* **14**, 57-66. (2004).